IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Surolia, N.	Examiner:	Weddington, K.
Serial Number:	09/763,499	Art Unit:	1614
Filing Date:	February 23, 2001	Attorney Docket:	2003710-0001 (IN99/00026)

Title: USE OF HYDROXYDIPHENYL ETHER CLASS OF CHEMICALS, AS EXEMPLIFIED BY TRICLOSAN, AS AN ANTIMALARIAL AND IDENTIFICATION OF FATTY ACID SYNTHESIS AS ITS TARGET

Mail Stop: AF
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

DECLARATION UNDER 37 C.F.R. § 1.132

I, Dr. Namita Surolia, declare as follows:

1. I am an inventor of the subject matter disclosed and claimed in United States patent application, Serial Number 09/763,499, filed February 23, 2001, and entitled "Use of Hydroxydiphenyl Ether Class of Chemicals, as Exemplified by Triclosan, as an Antimalarial and Identification of Fatty Acid Synthesis as its Target".

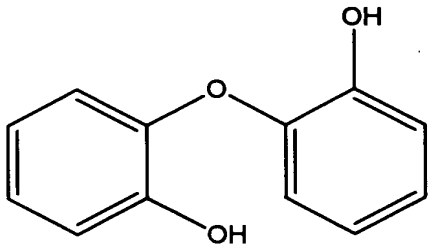
2. I am an Associate Professor in the Molecular Biology and Genetics Unit at the Jawaharlal Nehru Centre for Advanced Scientific Research is the assignee of the above-referenced patent application. My research focuses on the identification of parasite (*Plasmodium*) specific targets for drug development, the role of the plastid in *Plasmodium*, and detailed analysis of *Plasmodium* fatty acid synthesis. A copy of my *curriculum vitae* is attached hereto as **Appendix A**.

3. I have read the Office Action mailed August 15, 2005, and understand that the Examiner requests further evidence regarding the invention as claimed in the present application and specifically as it pertains to the ability of one of skill in the art to use inhibitors of fatty acid

synthesis, in particular members of the class of compounds known as hydroxydiphenyl ethers, as agents for the treatment of malaria.

4. As described in the instant patent application, my research group has previously shown that triclosan [5-chloro-2-(2,4-dichlorophenoxy)phenol], a hydroxydiphenyl ether, potently inhibits the growth of *Plasmodium falciparum in vitro* and, inhibits the growth of *Plasmodium berghei in vivo* in a mouse model. We reported these results in Surolia, N. and Surolia, A., *Nature Medicine*, 7(2), pp. 167-173, 2001.

5. In addition, my research group has shown that a second hydroxydiphenyl ether, namely 2,2' dihydroxydiphenyl ether, also inhibits *in vitro* growth of *Plasmodium falciparum*. The structure of this compound is shown below.



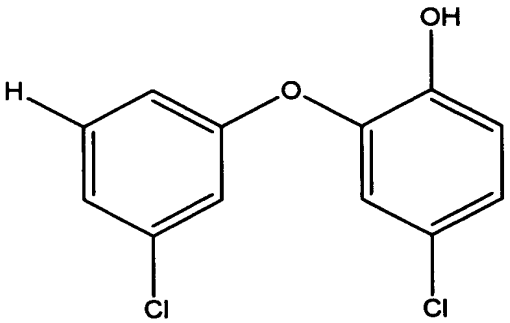
2,2' dihydroxydiphenyl ether

Specifically, the following experiment was performed in my laboratory and under my direction: We cultured *P. falciparum* strain FCK2 using standard techniques. *P. falciparum* growth was assessed by measuring the incorporation of [^{3}H] hypoxanthine with synchronized parasites at ring stage with 1-2% parasitemia, according to a method previously described in the literature (Ancelin, M.L., et al., *Blood* 91, 1426-1437, 1998). Aliquots of stock solution of 2,2' dihydroxydiphenyl ether were placed in tissue culture plates at a range of final concentrations in 0.005% DMSO after the addition of uninfected or infected red cell suspension in culture medium. The plates were placed in candle jars and incubated at 37°C for 4, 28, or 52 h for assessing the growth at 24, 48 and 72 h, respectively. [^{3}H] hypoxanthine (Amersham; 25.1 Ci/mmol, 5-20 μCi/ml, final concentration) was then added to each well at these time points (5% vol/vol) and after a further 20 h incubation, cells were collected and the radioactivity measured by liquid scintillation counting. The incorporation of [^{3}H] hypoxanthine reflects growth of the parasites and thus a reduced amount of radioactivity in cells from cultures that received 2,2' dihydroxydiphenyl ether

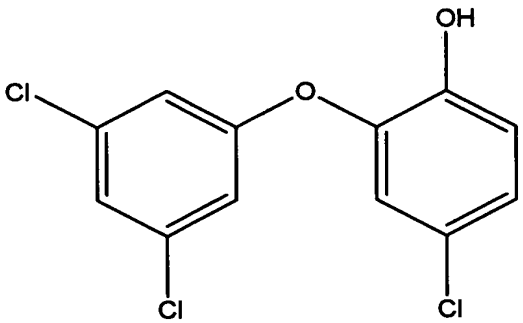
served as an indication that 2,2' dihydroxydiphenyl ether inhibited growth of the parasites. The IC_{50} value for 2,2' dihydroxydiphenyl ether was determined to be 1mM using this assay. As known in the art, in this context the IC_{50} value represents the concentration of a compound needed to inhibit growth by 50%, relative to a control culture in which the compound is absent. We reported these results in Surolia, N. and Surolia, A., *Nature Medicine*, 7(2), pp. 167-173, 2001.

6. We further showed that cerulenin (2,3-epoxy-4-oxo-7,10-dodecadienamide), a non-competitive inhibitor of fatty acid synthase that is structurally unrelated to hydroxydiphenyl ethers, inhibits growth of *Plasmodium falciparum in vitro*. The experiment was performed essentially as described in paragraph 5. The IC_{50} value for cerulenin was determined to be 20 μM. This result was also reported in Surolia, N. and Surolia, A., *Nature Medicine*, 7(2), pp. 167-173, 2001.

7. We have also shown that two additional members of the class of hydroxydiphenyl ether compounds, 4-chloro-2-(3-chloro-phenoxy)-phenol (referred to herein as compound A), and 4-chloro-2-(3,5-dichloro-phenoxy)-phenol (referred to herein as compound B), inhibit fatty acid synthesis in *Plasmodium*. The compounds were synthesized and purified in my laboratory and were characterized by ^{1}HNMR, ^{13}C NMR, and mass spectrometry, and their content of carbon, hydrogen, and nitrogen was determined by Pregl's method using a thermal conductivity detector. The structures of the compounds, as determined based on the foregoing analyses, are depicted below.



4-Chloro-2-(3-chloro-phenoxy)-phenol
Compound A



4-Chloro-2-(3,5-dichloro-phenoxy)-phenol
Compound B

8. The effect of Compounds A and B on fatty acid synthesis was determined by measuring the incorporation of [^{14}C] acetate into fatty acids in *P. falciparum* cultures. Various amounts of the

compounds were added to 200 ml cultures of *P. falciparum* that were then resuspended in 6.0 ml of the complete medium, while retaining the same concentration of the inhibitory compound. To this we added 1,2[^{14}C]acetate (50 μCi/ml, sodium acetate 60 mCi/mmol, NEN). After 2 h, parasites were isolated, washed thoroughly with PBS, lysed, sonicated, spotted onto a Whatman 3 MM paper disc, washed with TCA as described by Levy et.al. (McMurray, L.M., Oethinger, M. and Levy, S.B. : Nature 394, 531-532 ,1998) and counted in the scintillation fluid. The incorporation of 1,2[^{14}C]acetate was linear up to 4 h. We determined that the IC_{50} for fatty acid synthesis was 61.0 μM for compound A and 90.0 μM for compound B.

9. We have shown that compounds A and B inhibit *in vitro* growth of *P. falciparum*. The experiments were performed essentially as described in paragraph 5. The IC_{50} for compound A was 27.0 μM. The IC_{50} for compound B was 33.0 μM.

10. We have also shown that compounds A and B inhibit the growth of *Plasmodium berghei in vivo* in a mouse model. Specifically, the following experiment was performed in my laboratory and under my direction:

We determined the *in vivo* activity of the two compounds with a modified 4 day suppressive test (see Peters, W. in *Malaria*. (ed. Kreir, J.P.) (Academic Press, New York, 1980). Groups of BALB/c mice (6 mice per group) were intravenously inoculated with 1 x 10^7 infected erythrocytes on day 0. On day 1, infection was confirmed and the treatment was started. Various doses of compound A or compound B were administered subcutaneously once a day, over a period of 4 days, to groups of 6 mice. Control mice received no treatment. The survival of the mice was monitored.

Compound A was effective in extending survival of mice suffering from malaria caused by infection with *P. berghei* when administered at 250 mg/kg. The six control (untreated) mice died between day 5 and 7 (2 on day 5; 3 on day 6, and 1 on day 7). Mice treated with compound A survived for several additional days. All of the treated mice survived through day 5. Two died on day 6; 2 died on day 7; 1 died on day 9; and 1 survived for 2 weeks.

Compound B was also effective in extending survival of mice suffering from malaria caused by infection with *P. berghei* when administered at 250 mg/kg. The six control (untreated) mice died between day 5 and 7 (1 on day 5; 4 on day 6, and 1 on day 7). Mice treated with

compound B survived for a longer period of time, on average, than did untreated mice. All of the treated mice survived through day 5. Two died on day 6; 2 died on day 7; 1 died on day 10; and 1 survived for 3 weeks.

Thus both compounds A and B were effective in prolonging the average survival of mice infected with *P. berghei* following a period of treatment with the compound.

11. I, Namita Surolia, declare that all statements made herein of my own knowledge are true and that these statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful, false statements and the like are made punishable by fine or imprisonment, or both, under § 1001 of Title 18 of the United States Code and that such willful, false statements may jeopardize the validity of the application or any patents that may issue thereon.

Namita Surolia, Ph.D.

Date 6TH Feb. 2006

APPENDIX A

CV of Prof. Namita Surolia

Name: Namita Surolia **Position:** Associate Professor

Department/Institute/University: Molecular Biology and Genetics Unit
Jawaharlal Nehru Centre for Advanced Scientific Research
Bangalore 560064.

Date of Birth: 2nd April 1953 **Sex:** Female **SC/ST:** No

Education (Post-Graduation onwards & Professional Career)

Sl No.	Institution	Place	Degree Awarded	Year
1.	Allahabad University	Allahabad	M.Sc.	1975
2.	Allahabad University	Allahabad	Ph.D.	1979

Research Experience

University/Institution	Year(s)	Position held
Allahabad University	1974-77	JRF (UGC)
Kothari Scientific Research Institute, Calcutta	1977-79	SRF
Jadavpur University	1979-81	SRF(CSIR)
Indian Institute of Science	1982-87	Research Associate
Indian Institute of Science	1987-95	Senior Scientific Officer
Jawaharlal Nehru Centre for Advanced Scientific Research	1996-2002	Assistant Professor
Jawaharlal Nehru Centre for Advanced Scientific Research	2002- till date	Associate Professor

Honors and awards:

1. Recipient of VASVIK award for the year 2002.
2. Fellow, Indian Academy of Sciences, 2002.
3. Fellow, National Academy of Sciences, 2002.
4. Member, Guha Research Conference (GRC) India, 2001.
5. MOT Iyengar Award by Indian Council of Medical Research, for original contribution in medical sciences, 2002.
6. Delivered a Plenary lecture at 27th Annual Meeting on Basic Research in Chages Diseases and the XVII Annual Meeting of the Brazilian Society at Caxambu, Brazil (2001).
7. Delivered a plenary lecture at the Malaria Medical Ventures (WHO) and Confederation of Indian Industry International Summit on Medicines for Malaria at New Delhi (2002).
8. Invited speaker at COST B9 Congress at London, U.K. (2002).
9. Invited speaker at workshop organized by the Africa Human Genome initiative, Cape Town, South Africa (19th -22nd March 2003).
10. Invited speaker at FAOBMB Congress, Thailand (2004).
11. Delivered invited lecture, organized by the Africa Human Genome Institute, Nairobi, Kenya, South Africa (22nd- 25th March 2005).
12. S.D. Srivastava Gold Medal for the outstanding academic performance during B.Sc. by University of Allahabad (1971).
13. Best female student award from the Science Faculty, University of Allahabad, 1975.
14. JRF (UGC) 1974-77 & SRF (CSIR) 1979-81.
15. S.R.F. awarded by Kothari Scientific Research Institute, Calcutta, 1977-79.

16. IUB travel fellowship award by IUB for attending 13th International Congress of Biochemistry at Amsterdam (August 1985).

Additionally delivered about 50 invited talks and chaired several sessions in various National and International conferences held within India.

Publications (Numbers only)

Books: Nil **Research Papers:** 43 **General articles:** 2

List of important publications.

1. NMR assignment of the holo-ACP from malaria parasite *Plasmodium falciparum*, Sharma, A.K. Sharma, S.K, Surolia, N., Sarma P.S. **J. Biomol. NMR.** 2005 (in press).

2. A novel approach for over-expression, characterization, and isotopic enrichment of a homogeneous species of acyl carrier protein from *Plasmodium falciparum*. Sharma SK, Modak R, Sharma S, Sharma AK, Sarma SP, Surolia A, Surolia N. **Biochem Biophys Res Commun**. 2005 May 20;330(4):1019-26.

3. Production and purification of refolded recombinant *Plasmodium falciparum* beta-ketoacyl-ACP reductase from inclusion bodies. Karmodiya K, Srivastav RK, Surolia N **Protein Expression and Purification**, April 2005.

4. Structural basis for the variation in triclosan affinity to enoyl reductases. Pidugu LS, Kapoor M, Surolia N, Surolia A, Suguna K. **J Mol Biol.** 2004 Oct 8; 343(1):147-55

5. 'Fas't inhibition of malaria: Surolia A, Ramya TN, Ramya V, Surolia N **Biochem J. (Review)** (2004); 383(Pt. 3):401-12.

6. Mutational analysis of the triclosan-binding region of enoyl-ACP (acyl-carrier protein) reductase from *Plasmodium falciparum* Kapoor, M, Gopalakrishnapai, J, Surolia, N, Surolia, A **Biochem J.** 2004 Aug 1;381(Pt 3):735-41.

7. Kinetic and structural analysis of the increased affinity of enoyl-ACP reductase for triclosan in the presence of NAD Kapoor, M, Mukhi PL, Surolia N, Suguna K, Surolia A. **Biochem J.** 2004 Aug 1;381(Pt 3):725-33.

8. Slow-tight binding inhibition of enoyl-acyl carrier protein reductase from *Plasmodium falciparum* by triclosan. Kapoor M, Reddy CC, Krishnasastry MV, Surolia N, Surolia A. **Biochem J**. 2004 Apr 16; 381: 1-6.

9. Crystallization and preliminary crystallographic analysis of beta-hydroxyacyl ACP dehydratase (FabZ) from *Plasmodium falciparum*. Lakshmi Swarna Mukhi P, Kumar Sharma S, Kapoor M, Surolia N, Surolia A, Suguna K. **Acta Crystallogr D Biol Crystallogr.** 2004 Jan;60(Pt 1):120-1.

10. Triclosan as a Systemic Antibacterial Agent in an Acute Bacterial Challenge Mouse Model Sharma et al. **Antimicrobial Agents and Chemotherapy** (2003) Dec;47(12):3859-66.

11. Triclosan: A shot in the Arm for Antimalarial Chemotherapy: Satish P. RamachandraRao, Namita and Avadhesha Surolia **Molecular and Cellular Biochemistry** (2003) Nov;253(1-2):55-63.

12. Identification, Characterization and Inhibition of *Plasmodium falciparum* β-Hydroxyacyl-Acyl Carrier Protein Dehydratase (FabZ): Shailendra Kumar Sharma, Mili Kapoor, T.N.c. Ramya, Sanjay Kumar, Gyanendra Kumar, Rahul Modak, Shilpi Sharma, Namita Surolia and Avadhesha Surolia (2003) **J. Biol. Chem**. 278, 45661-45671.

13. Functional characterization of β-ketoacyl-ACP reductase (FabG) from *Plasmodium falciparum,* Smitha Pillai Chitra Rajagopal, Mili Kapoor, Gyanendra Kumar, Aditi Gupta, and Namita Surolia (2003), **BBRC**, 303, 387-392.

14. Exploring the interaction energies for the binding of hydroxydiphenyl ethers to enoyl-acyl carrier protein reductases: Jayaraman Muralidharan, Kaza Suguna, Namita Surolia and Avadhesha Surolia(2003) **J. Biomol. Struct. Dyn**. 20(4), 589-94.

15. Paradigm shifts in malaria parasite biochemistry and antimalarial chemotherapy: Namita Surolia, Satish P. Rao and Avadhesha Surolia (2002) **BioEssays** 24.2, 192-196.

16. Survival Strategies of the malarial parasite *Plasmodium falciparum:* T.N.C. Ramya, Namita Surolia and Avadesha Surolia (2002) **Current Science** 83, 101-108.

17. Structural basis for triclosan and NAD binding to enoyl-ACP reductase of *Plasmodium falciparum:* Kaza Suguna, Avadhesha Surolia and Namita Surolia (2001) **Biochem. Biophys. Res. Commun.** 283, 224-228.

18. Kinetic determinants of the interaction of enoyl-ACP reductase from *Plasmodium falciparum* with its substrates and inhibitors: Mili Kapoor, M. Jamal Dar, Avadhesha Surolia and Namita Surolia (2001) **Biochem. Biophys. Res. Commun.** 289, 832-837.

19. Triclosan offers protection against blood stages of malaria by inhibiting enoyl-ACP reductase of *P. falciparum*: Surolia N and Surolia A **Nature Medicine** 2001; 7, 167-173.

20. *In vivo* antimalarial activity of extracts of three plants used in the traditional medicine of India. G. Praveen Bhat and Namita Surolia (2001) **Am. J. Trop. Med. Hyg.** 65(4), 304-308.

21. Triclosan and fatty acid synthesis in *Plasmodium falciparum:* A new weapon, a new target and an old enemy: Praveen Bhat G. and Namita Surolia (2001) **J. Biosci.** 26, 1-3.

22. Chloroquine binds in the cofactor binding site of *Plasmodium falciparum* lactate dehydrogenase: Namita Surolia (2000) **Parasitology Today** 16, 133.

23. Receptor mediated targeting of toxins to intraerythrocytic parasite *Plasmodium falciparum:* Namita Surolia (2000) **Advanced Drug Delivery Reviews** 41, 163-170.

24. Interaction of chloroquine and its analogues with heme: An isothermal titration calorimetric study: Bachhawat, K., Thomas, C.J., Surolia, N. Surolia, A. (2000) **Biochem. Biophys. Res. Commun.** 276, 1075-9.

25. Cell surface receptor directed targeting of toxins to *Plasmodium falciparum:* Surolia, N. and Misquith, S. (1996) **FEBS Lett.** 396, 57-61.

26. *De novo* biosynthesis of heme in *Plasmodium falciparum:* Surolia, N. (1996) **Parasitology Today** 12, 495.

27. Molecular basis of chloroquine resistance in the malarial parasite in Drug Resistance: Mechanism and Management: Karthikeyan, G., Bonday, Z.Q., Surolia N. and Padmanaban, G. (1997) Editors: Singhal, R.L. and Sood, O.P. Publishers: Ranbaxy Science Foundation. P15-21.

28. Involvement of cytochrome p-450 in conferring chloroquine resistance to the malarial parasite, *Plasmodium falciparum:* Namita Surolia, G. Karthikeyan and G. Padmanaban (1993) **Biochem. Biophys. Res. Commun.** 197, 562-569.

29. De novo biosynthesis of heme offers a new chemotherapeutic target in the human malarial parasite: Namita Surolia and G. Padmanaban (1992) **Biochem. Biophys. Res. Commun.** 187, 744-750.

30. Chloroquine inhibits heme-dependent protein synthesis in *Plasmodium falciparum:* Namita Surolia and G. Padmanaban (1991) **Proc. Natl. Acad. Sci. USA** 88 4786-4790.

b. Publications in the area of endotoxic shock:

31. Kinetic and thermodynamic analysis of the interactions of 23 residue peptides with endotoxin: Celestine J. Thomas, Namita Surolia and Avadhesha Surolia (1999) **J. Biol. Chem.** 276, 35701-35706.

32. Surface plasmon resonance studies resolve the enigmatic endotoxin neutralizing activity of polymyxin B: Celestine J. Thomas, Namita Surolia and Avadhesha Surolia (1999) **J. Biol. Chem.** 274, 29624-29627.

31. Kinetics and mechanism of the recognition of endotoxin by Polymyxin B: C.J. Thomas, B.P. Ganghadhar, N. Surolia and A. Surolia. (1998) **J. Am. Chem. Soc.** 120, 12628-12634.

32. Titration calorimetric studies to elucidate the specificity of the interactions of polymyxin B with lipopolysaccharides and lipid A: Srimal, S., Surolia, N., Balasubramanian, S. and Surolia, A. (1996) **Biochem. J.** 315, 679-686.

c. Publications on protein-carbohydrate interactions:

33. One step synthesis of 2-hydroxydiarylether by using domestic microwave heating: Sanjay Kumar, Mili Kapoor, Namita Surolia and Avadhesha Surolia (2003) **Synthetic Commun.** 34(3). 413-420.

34. Plasticity in the primary binding site of galactose/N-acetylgalactosamine specific lectins. Implication of C-H...O hydrogen bond at the specificity determining C4 locus of the saccharide in 4-methoxygalactose recognition by Jacalin and WBAI: Chittor P. Swaminathan, Aditi Gupta, Namita Surolia and Avadhesha Surolia (2000) **J. Biol. Chem.** 275, 28483-28487.

35. Role of water in the specific binding of mannose and mannooligosaccharides to Concanavalin A: Chittor P. Swaminathan, Namita Surolia and Avadhesha Surolia (1998) **J. Am. Chem. Soc.** 120, 5153-5159.

36. On the relationship of Thermodynamic Parameters with the Buried Surface Area in Protein-ligand Complex Formation: Sigha, N., Surolia, N. and Surolia, A. (1996) **Bioscience Reports** 16(1), 1.

37. Binding of 4-Methylumbelliferyl-β-D-galactopyranoside to *Momordica charantia* lectin: A fluorescence quenching study. M.I. Khan, T. Majumdar, D.K. Pain, N. Gaur & A. Surolia (1981) **Eur. J. Biochem.** 113, 471.

38. Purification and characterization of galactose binding lectin *Momordica charantia*: T. Majumdar, N. Gaur & A. Surolia (1981) **Eur. J. Biochem.** 113, 463.

39. Binding of 4-methyllumbelliferyl-β-D-galactopyranoside to *Abrus precatorius* lectin: A fluorescence, quenching and polarization studies: M.I. Khan, M.K. Mathew, N. Surolia, P. Balaram & A. Surolia (1981) **Eur. J. Biochem.** 115, 249.

d. Publications in the area of riboflavin carrier protein and flavoenzymes

40. Mechanism of foetal wastage following immunoneutralization of riboflavin carrier protein in the pregnant rat: disturbance of flavin coenzyme levels: K. Krishnamurty, N. Surolia & P.A. Adiga (1985) **FEBS Lett.** 178, 87.

41. Enzymic basis of deranged foetal flavin-nucleotide metabolism consequent on immunoneutralization of maternal riboflavin carrier protein in the pregnant: N. Surolia, K. Krishnamurthy & & P.A. Adiga (1985) **Biochem. J.** 230, 365-367.

e. Publications in the field of nitrogen fixation.

42. Effect of amino acids on microbial fixation of nitrogen: Krishna Bahadur and Namita Gaur (1980) **Zentralblatt fur baktriologie and Hygiene II Abt** 135, 674-681.

43. Effect of Ruthenium on nitrogen fixation by some nitrogen fixers: Krishna Bahadur & Namita Gaur (1979) **Zentralblatt fur baktriologie, Parasiten Kunde. Infections Krankheiten and Hygiene. Abt.** 134, 594-599.